Somoza *et al. Biology Direct* (2024) 19:67
https://doi.org/10.1186/s13062-024-00501-1

Biology Direct

REVIEW

Open Access

Breaking barriers: improving time and space resolution of arbuscular mycorrhizal symbiosis with single-cell sequencing approaches

Sofía Cristina Somoza[1], Paola Bonfante[2] and Marco Giovannetti[1,2*]

Abstract

The cell and molecular bases of arbuscular mycorrhizal (AM) symbiosis, a crucial plant-fungal interaction for nutrient acquisition, have been extensively investigated by coupling traditional RNA sequencing techniques of roots sampled in bulk, with methods to capture subsets of cells such as laser microdissection. These approaches have revealed central regulators of this complex relationship, yet the requisite level of detail to effectively untangle the intricacies of temporal and spatial development remains elusive.

The recent adoption of single-cell RNA sequencing (scRNA-seq) techniques in plant research is revolutionizing our ability to dissect the intricate transcriptional profiles of plant-microbe interactions, offering unparalleled insights into the diversity and dynamics of individual cells during symbiosis. The isolation of plant cells is particularly challenging due to the presence of cell walls, leading plant researchers to widely adopt nuclei isolation methods. Despite the increased resolution that single-cell analyses offer, it also comes at the cost of spatial perspective, hence, it is necessary the integration of these approaches with spatial transcriptomics to obtain a comprehensive overview.

To date, few single-cell studies on plant-microbe interactions have been published, most of which provide high-resolution cell atlases that will become crucial for fully deciphering symbiotic interactions and addressing future questions. In AM symbiosis research, key processes such as the mutual recognition of partners during arbuscule development within cortical cells, or arbuscule senescence and degeneration, remain poorly understood, and these advancements are expected to shed light on these processes and contribute to a deeper understanding of this plant-fungal interaction.

Keywords Arbuscular mycorrhizal symbiosis, Single-cell sequencing, Single-nucleus sequencing, Plant-microbe interactions, Spatial transcriptomics, Plant cell atlas

*Correspondence:
Marco Giovannetti
marco.giovannetti@unito.it
[1]Department of Biology, University of Padova, Padova 35131, Italy
[2]Department of Life Sciences and Systems Biology, University of Torino, Torino 10125, Italy

Background

Plants never live alone. Arbuscular mycorrhizae (AM) offer one of the most powerful examples of the interaction capacity of plants to associate with the phylogenetically distant kingdom of fungi. Mycorrhizal symbioses have been known since the end of the 19th century [1], but the understanding of the AM success in space, being present in more than 72% of current land plants [2], and in time, dating back to 400 MYAs ago, is more recent. Physiological, genetic, molecular biology and -omics approaches have demonstrated some mechanisms which are at the basis of the AM success, such as the exchange of nutrients between the partners. The reduced carbon by the plant flows towards the fungus, which in turn improves the mineral nutrition of its green host [3], leading to a root-shoot axis which often has systemic positive effects [4]. The molecular dialogue and the mutual regulation between the two partners have revealed a surprising intimate interaction [5, 6], where the fungal role remains more enigmatic, even if the genomes of many AM fungi have been sequenced [7].

The benefits of the AM symbiosis are so important that international agencies consider them in the context of safeguarding the planet's health, preserving soil fertility and pushing towards more sustainable agriculture. However, the steps towards an application still have many obstacles: the systems that are studied in laboratories are very few in comparison to the huge biodiversity found in nature, or even just in agroecosystems. Furthermore, advanced biology that integrates genetic approaches and -omics techniques usually operates on the root system of AM plants. On the contrary, we know that recognition processes occur within the rhizosphere, involving strigolactones (SL) and short-chain chitin oligomers (COs) exuded by the root and fungus respectively [8, 9]. The fungus uptakes mineral elements thanks to its extraradical hyphae, but it is also capable of penetrating the root by the hyphopodium to reach the cortical cells, where throughout hyphal branching, arbuscules are formed. Even if the arbusculated hyphae are found in close proximity with the cytoplasm of the host cells, they remain physically separated by the peri-arbuscular membrane, in continuity with the plant cell plasma membrane, and establishing an extensive surface area where nutrient exchanges occur [10, 11] (Fig. 1). Altogether, it is clear that symbiotic functions are compartmentalised with a specific role for each cell type. These concepts already emerged from transmission electron microscopy studies in the 80–90s [1] which showed the different reactivity of root cells to the advancing fungus as well as

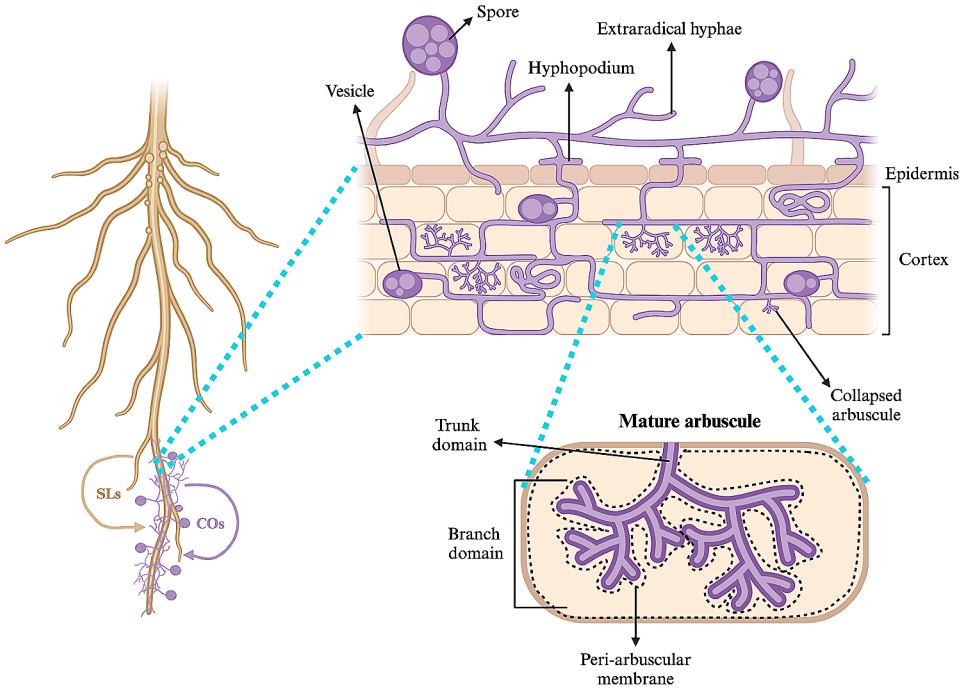

Fig. 1 Root colonization by AM fungi. Plants recognize fungal molecules, such as short-chain chitin oligomers (COs) or Myc factors, found in AM fungi exudates; in turn, plant strigolactones (SLs) induce spore germination and hyphal branching. Upon initial contact with the root epidermis, tip-growing hyphae differentiate, forming a structure known as a hyphopodium. Subsequently, the fungus penetrates the root guided by a plant structure called the pre-penetration apparatus (PPA). Once inside the root, the hypha traverses intercellular spaces of the inner cortex root cells and, following PPA formation, enters the cell and initiates the trunk formation. Then, young arbuscules are produced, which expand within the cortical cell through continuous branching until mature, occupying the entire space and engaging in mutualistic nutrient exchanges. These mature functional arbuscules persist for several days before undergoing senescence. Created with BioRender.com

the progressive changes of the latter when crossing the root tissues. Ultrastructural observations coupled with in situ labelling techniques allowed to describe changes in the fungal wall mirroring structural chitin modifications, when moving from the extraradical hyphae to the arbuscules [12, 13]. Looking at the plant side, conspicuous molecular changes were detectable in the plant material laid down at the partner interface by using a range of monoclonal antibodies and depending on the root cell typology [14] as well as in the fine nuclear organization [15]. Lastly, the discovery of endobacteria living inside AM hyphae [16] has opened the way to the novel concept of the fungal microbiota [17].

With the current advances in molecular biology, molecular and cellular knowledge therefore necessitates a deeper integration also in the field of plant-microbe interactions. The first aim of this review is to highlight how some techniques already developed in the past (laser microdissection, confocal studies by using plant lines transformed with reporter genes, etc.) have given relevant contributions by highlighting the characteristics of colonized cells versus non-colonized cells within the same AM root. The second objective is to illustrate how new technologies such as single-cell RNA sequencing (scRNA-seq), that recently started to be applied in the study of some plant-microbe interactions, can open new horizons and new questions to better dissect and appreciate the potentiality of the AM symbiosis process.

How technologies have shaped the cellular resolution of arbuscular mycorrhizal association

Arbuscular mycorrhizas, as other plant-microbe systems, illustrate how the development of molecular technologies, beginning with the PCR, paved the way for crucial discoveries. Experimental limitations given by the obligate symbiotic nature of AM fungi were overcome only at the end of the 90s, opening a new era based on molecular and genetic analyses.

Starting from that period, the establishment of new techniques led to crucial discoveries of AM symbiosis functioning coupled with a cellular resolution gain. It has been known for a long time that plants activate a specific phosphate mycorrhizal pathway [18], but only through testing promoter activity with histochemical GUS approaches [19] and protein localization with confocal microscopy it was possible to describe the location of plant phosphate transporters. Many research groups [20–23] demonstrated the dominant expression of AM-induced phosphate transporters in the root cortical cells containing arbuscules, and more precisely on the peri-fungal membrane which always surrounds the intracellular fungus [11] (Fig. 1).

Similarly, our capacity to follow in vivo single-cell development and rearrangements of plant cellular compartments during the different steps of AM formation has shaped our understanding of this process. Live cell imaging of intraradical hyphal growth using fluorescent markers of the cellular endomembrane system has brought to the identification of a novel plant structure, the plant pre-penetration apparatus [24, 25], and allowed to follow the intricate vesicle trafficking orchestrating the substantial reshaping of the cellular environment [26].

Later on, being able to observe live plant cells hosting fungal arbuscules in greater detail allowed researchers to identify two distinct domains within the peri-arbuscular membrane surrounding the arbuscule-hosting cells, harbouring different sets of proteins and mediating different processes [13]: the trunk domain, which represents the base of the arbuscule and accumulates similar proteins to the ones found in the peripheral plasma membrane, and the branch domain, that encases the fungal hyphal branches and contains specific proteins primarily needed for plant-fungus signalling and nutrient exchange [27] (Fig. 1).

Because mycorrhizal colonization occurs asynchronously, a bulk characterization of whole roots provides an average representation of all developmental stages and cell-types present in a single root (Fig. 2). Hence, numerous studies have employed laser microdissection methodologies [28–39] to gain more comprehensive insights into gene expression associated with various stages and cell-types during this interaction. This approach was introduced back in 1976 by pathologists to cut single cells from freeze-dried tissue [40], and applied for the first time in plant tissues 16 years later [41]. Combined with RT-qPCR or microarray to have a genome-wide picture of gene regulation, the laser microdissection technology has surely contributed to the identification of a core set of genes that are regulated by AM interaction and preferentially active in arbusculated cells: among them, most of the so-far identified plant nutrient transporters, such as the iconic phosphate transporter, an ammonium and a sulphate transporter, as well as a fungal inorganic phosphate (Pi) transporter, previously hypothesized to be present exclusively in the extraradical hyphae and fungal-enriched RNA [42]. However, laser microdissection as a method is limited to the selected cells and does not easily differentiate among the diverse coexistent arbuscular stages.

From whole-root to single-cell RNA sequencing: opportunities and challenges

Among the new technologies that enriched plant research in the past few years, transcriptomics stands out, expanding into multiple fields in biology and being applied to numerous organisms. Particularly, when studying plant-microbe interactions, both symbiotic partners create a multi-factorial transcriptomic and

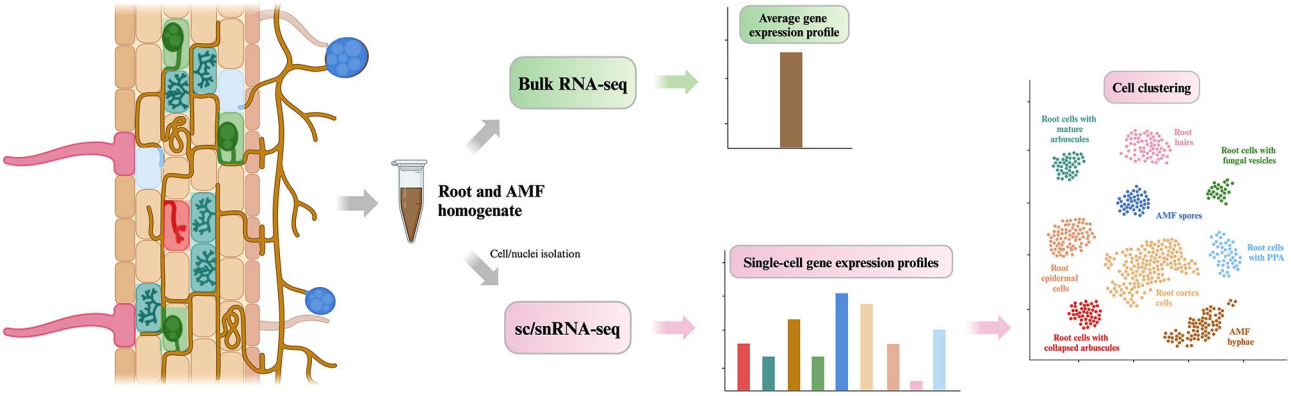

Fig. 2 Bulk versus sc/sn RNA sequencing in mycorrhizal roots. Bulk RNA sequencing offers an average gene expression profile of all the root and fungal cells. On the other hand, single-cell and single-nucleus RNA sequencing offer a distinct transcriptome profile for each individual plant and fungal cell, enabling the grouping of the different cell populations into physiological or functional clusters. Created with BioRender.com

proteomic environment [43]. In this context, traditional methods such as bulk RNA-seq and microarrays served as primary techniques for profiling the transcriptome but merely depicted the collective transcriptional patterns averaged across a population of thousands of cells. Single-cell RNA sequencing, as the name states, embraces different techniques used for sequencing the RNA present in individual cells, which means the sequencing of a single transcriptome and gene expression analysis in each cell in an independent way, overcoming the obstacles of bulk RNA-seq (Fig. 2).

The first step to carry out a single-cell study is the dissociation of the cells from the plant tissue. One key attribute of plant cells that has to be considered within this step is the presence of the structural carbohydrate-rich cell wall surrounding the plasma membrane. To isolate plant cells, the cell wall must be removed through enzymatic digestion, a procedure known as protoplasting, which yields single cells without cell walls (protoplasts) [44]. However, this process is often associated with technical challenges, like incomplete cell isolations as a result of the wide range of cell sizes and cell wall compositions, or the position of the layers through the tissue [45], resulting in limited access to certain cellular sub-populations and, in consequence, to a detection bias toward easier-to-protoplast cell-types [46, 47], which could represent an obstacle if the objective is to perform cell annotation [48, 49]. In addition, protoplasting can be difficult or impractical to apply in certain plant tissues or species with diverse cell wall components that need a particular optimization [50, 51]; still, the main problem associated with the protoplasting methods in transcriptomics research is its impact on gene expression due to the introduction of enzymes that can develop plant stress responses or cause mRNA leaking [50, 52]. To overcome these challenges associated with cell isolation, single-nucleus RNA-sequencing (snRNA-seq), which requires nuclei isolation, has lately become a promising alternative to use in plant research as a protoplasting-free method.

Even in other fields that do not involve plants or cellulose cell walls, like mammalian research, the implementation of snRNA-seq has largely increased over the last years due to the advantages that this technique shows for certain aims, like getting the expected cell-type frequencies and the limitations found in scRNA-seq [53]. Moreover, protein-coding genes and long non-coding RNAs are highly detected with snRNA-seq, as well as genes with a large intronic burden, likely because these require more time within the nucleus for an accurate splicing. However, it has to be considered that the amount of data obtained per nucleus is from 3 to 10 times lower than the one obtained per cell through scRNA-seq [53–55]. In addition, if the study aims to understand plastid or mitochondrial gene expression, scRNA-seq would represent a better choice.

Alongside the decision between cells or nuclei isolation, there are several strategies for its capture that can be used, intending to physically separate them from the others. Currently, the technology most commonly utilized for single-cell capture in plants is based on droplet microfluidics, which revolutionized single-cell studies by significantly increasing the number of cells that can be profiled in comparison to classic plate-based assays or laser microdissection [56]; in fact, all the plant-microbe interaction studies published until now and reviewed here (Table 1) employ the droplet microfluidics-based 10X Genomics Chromium system, which consists in encapsulating cells/nuclei in single gel beads containing barcoded oligonucleotides that label the RNA molecules through reverse transcription and produce a library for sequencing through next-generation sequencing (NGS) platforms [57, 58]. Nevertheless, new technologies based on combinatorial barcoding that, unlike droplet microfluidic-based methods, do not require complex partitioning equipment or a combination of both approaches,

Table 1 An overview of single-cell research studies in plant-microbe interactions ordered by publication time. scRNA-seq = single-cell RNA sequencing, snRNA-seq = single nucleus RNA sequencing, snATAC-seq = assay for transposase-accessible chromatin using single nucleus sequencing, MERFISH = multiplexed error-robust fluorescence in situ hybridization

Title	Citation	Main technique/s	Results	Organisms
Spatial co-transcriptomics reveals discrete stages of the arbuscular mycorrhizal symbiosis	[76]	snRNA-seq and spatial transcriptomics	Construction of the first spatially-resolved single-cell resolution integrated map of a multi-kingdom symbiotic interaction. First single-cell resolution study of the AM symbiosis.	*Medicago truncatula*/ *Rhizophagus irregularis*
The single-cell transcriptome program of nodule development cellular lineages in *Medicago truncatula*	[60]	scRNA-seq	Description of cell-type-specific transcriptome response of *Medicago truncatula* roots to rhizobia during early nodule development.	*Medicago truncatula*/ *Sinorhizobium meliloti*
Spatial metatranscriptomics resolves host–bacteria–fungi interactomes	[80]	Spatial metatranscriptomics	Detection of spatial interaction between the plant and its colonizing microorganisms (55 µm of resolution). Study of bacterial and fungal hotspots and the plant's response.	*Arabidopsis thaliana*/ *Pseudomonas syringae*
Single-cell analysis identifies genes facilitating rhizobium infection in *Lotus japonicus*	[74]	scRNA-seq	Generation of wild type and mutant *L. japonicus* seedlings cell atlas after rhizobial infection. Identification of candidate nodulation genes.	*Lotus japonicus*/ *Mesorhizobium loti*
Cell-type-specific responses to fungal infection in plants revealed by single-cell transcriptomics	[112]	scRNA-seq	Generation of Arabidopsis cell atlas during fungal infection. Description of cell-type specific gene expression, like high expression of immune receptors in vasculature cells and coordinated expression changes of abscisic acid in the guard cells. Report of a robust correlation between cell response and its proximity to the invading fungal hyphae.	*Arabidopsis thaliana*/ *Colletotrichum higginsianum*
Single-nucleus transcriptomes reveal spatiotemporal symbiotic perception and early response in *Medicago*	[75]	snRNA-seq	Time-course analysis of symbiotic perception, focusing on early cell-type specific responses to nodulation.	*Medicago truncatula*/ *Sinorhizobium meliloti*
Cell specialization and coordination in *Arabidopsis* leaves upon pathogenic attack revealed by scRNA-seq	[77]	scRNA-seq	Analysis of the cellular heterogeneity within an infected leaf. Description of spatial and temporal dynamics of immune and susceptible cell-clusters.	*Arabidopsis thaliana*/ *Pseudomonas syringae*
Single-cell profiling of *Arabidopsis* leaves to *Pseudomonas syringae* infection	[78]	scRNA-seq, transcriptional reporter fusion	Analysis of the cellular heterogeneity within an infected leaf. Description of spatial and temporal dynamics of immune and susceptible cell-clusters.	*Arabidopsis thaliana*/ *Pseudomonas syringae*
Single-cell RNA sequencing profiles reveal cell type-specific transcriptional regulation networks conditioning fungal invasion in maize roots	[113]	scRNA-seq	Study of the immune regulatory networks in major cell types of maize root tips in response to fungal infection.	Maize (*Zea mays*)/ *Fusarium verticillioides*
Time-resolved single-cell and spatial gene regulatory atlas of plants under pathogen attack	[79]	snMultiome (snRNA-seq + snATAC-seq) and spatial transcriptomics (MERFISH)	Generation of a spatiotemporal cell map of Arabidopsis leaf infected by virulent and avirulent bacteria, involving transcription factors, putative cis-regulatory elements and target genes associated with disease and immunity. Identification of new cell populations involved in immune response. Discovery of immune response heterogeneity according to pathogen distribution.	*Arabidopsis thaliana*/ *Pseudomonas syringae*
A high-resolution transcriptomic atlas depicting nitrogen fixation and nodule development in soybean	[72]	snRNA-seq, transcriptional reporter fusion	Generation of cell atlas of soybean roots and determinate nodules. Comparison of determinate and indeterminate nodules. Description of *GmCRE1* role in nodule formation and nitrogen fixation.	Soybean (*Glycine max*)/ *Sinorhizobium fredii*
Integrated single-nucleus and spatial transcriptomics captures transitional states in soybean nodule maturation	[114]	snRNA-seq and spatial transcriptomics	Generation of cell atlas of soybean roots and determinate nodules. Identification of rare-cell subtypes with important roles in nodule function and development.	Soybean (*Glycine max*)/ *Bradyrhizobium diazoefficiens*
Single-cell resolution transcriptome atlases of soybean root organs reveal new regulatory programs controlling the nodulation process	[73]	snRNA-seq	Generation of cell atlas of soybean roots and determinate nodules. Identification of different subpopulations of *B. diazoeciens*-infected cells in the nodule. Characterization of GmFWL3 protein.	Soybean (*Glycine max*)/ *Bradyrhizobium diazoefficiens*

Table 1 (continued)

Title	Citation	Main technique/s	Results	Organisms
Single-cell transcriptomic analyses reveal cellular and molecular patterns of rubber tree response to early powdery mildew infection	[115]	scRNA-seq, transcriptional reporter fusion	Study of the distinct gene expression patterns of cell clusters under powdery mildew infection. Report of *HbCNL2* gene as disease-resistance gene.	Rubber tree (*Hevea brasiliensis*)/ *Oidium heveae*
Cell-specific pathways recruited for symbiotic nodulation in the *Medicago truncatula* legume	[71]	snRNA-seq	Analysis of the transcriptomic response of Medicago root cells to rhizobial infection.	*Medicago truncatula*/ *Ensifer meliloti*
Differentiation trajectories and biofunctions of symbiotic and unsymbiotic fate cells in root nodules of *Medicago truncatula*	[70]	scRNA-seq, transcriptional reporter fusion	Generation of single-cell transcriptome map of indeterminate root nodules.	*Medicago truncatula*/ *Sinorhizobium meliloti*
Development of a single-cell atlas for woodland strawberry (*Fragaria vesca*) leaves during early *Botrytis cinerea* infection using single cell RNA-seq	[69]	scRNA-seq	Generation of the first single-cell atlas of the plant pathogenic invasion process.	Woodland strawberry (*Fragaria vesca*)/ *Botrytis cinerea*

have been described in the past years [59] and are nowadays available as commercial kits, which could promote the accessibility to single-cell resolution research.

Typically, the first step in single-cell data analysis is to cluster and annotate the cells based on their similarities in gene expression profiles. To this aim, researchers focus on well-known specific cell-type marker genes, which exhibit high expression within a particular cell-type, and low or not expression in the others. However, the requirement of cells or nuclei isolation to perform sc/snRNAseq entails a loss of spatial perspective regarding cell location, and this challenge is just partially solved after bioinformatics processing for clustering. Tissues are composed of a multitude of cells with a precise spatial organization, a critical feature for executing their functional roles. Clustering methods neglect cells' spatial coordinates, not being able to differentiate spatially diverse cells that show similar transcriptomes. As a matter of fact, Pereira and colleagues [60] analyzed *M. truncatula* early nodule development and found a subset of cells in the nodule meristem cluster that was also present in non-infected plants. This suggested that those were likely cells from the lateral root meristem. In addition, when a plant-microbe interaction is being studied, the cell isolation means the displacement of the physical interaction between the partners.

Therefore, the need to understand cellular gene expression while investigating its spatial environment within a complex organ or tissue has prompted the development of a group of techniques known as spatial transcriptomics (ST) [61, 62]. These technologies are already extensively applied in animal research, but have only recently started to be adopted in the plant research field. Depending on how spatial information is acquired, ST methods can be divided into three groups: (i) laser capture microscopy-based methods, as LCM-seq, where individual or group of cells are harvested directly from the tissue's region of interest and then gene expression analyses are performed, (ii) imaging-based methods, that consist in in situ hybridization followed by the identification of the target RNAs through high resolution microscopy, like MERFISH (Multiplexed Error Robust Fluorescence In Situ Hybridization), a highly multiplexed single-molecule imaging approach that can detect thousands of RNA species at a single-cell resolution [63], and (iii) in situ capture sequencing-based methods, in which the location of the transcripts of interest is obtained via high-throughput sequencing, e.g. 10x Visium [64], with the last one representing a powerful tool to investigate a plethora of plant models and biological questions, as it combines spatial information with transcriptomic data; recently, You and collaborators [65] compared 11 sequencing-based spatial transcriptomics methods using animal reference tissues and explain how resolution is affected by molecular diffusion.. The progression of technology has facilitated the application of these techniques in studying the development of plant organs [66] or in spatially describing metabolic pathways [67] and, subsequently, researchers are delving into investigating plant-microbe interactions.

In summary, the single-cell resolution is transforming the quality and depth of the results obtained in the plant-microbe interaction field, especially when combined with ST approaches (Table 1). However, the conventional bulk RNA-seq methodology is indeed significantly more affordable, the data analysis is less complex and it presents a high sensitivity and little genome amplification required, reducing noise and biases. So, in the end, the choice of approach would depend on the particular objective of the study and the practical considerations, like the budget available or the characteristics of the samples [48, 68].

Single-cell level insights into plant-microbe interaction

In early 2022, Bai and colleagues [69] pioneered the first single-cell scale research of a plant-microbe interaction, delving into the infection of woodland strawberry

leaves by the fungus that causes grey mold disease, a significant threat in agriculture. They generated a high-resolution transcriptome atlas through scRNA-seq. Subsequently, they examined the individual responses of each cell-type to the infection, identifying differentially expressed genes, particularly those associated with stress response and secondary metabolism. Notably, the hydathode, upper epidermal and mesophyll cells showed the most robust initial responses to the infection. Altogether, each cell-type showed a specific expression pattern in the transcriptional regulation process, forming a sophisticated regulatory network to respond to fungal infections.

Nearly one year later, two groups generated the first single-cell resolution studies of the nodulation process, using the model legume *M. truncatula* [70, 71]. Both studies illuminated the impact of nodulation on the different cell-types, whether in the entire root [71] or focusing exclusively on nodule tissue cells [70], providing crucial resources and insights for advancing rhizobial nodulation research. Cervantes-Pérez and colleagues [71] studied the early response to the bacterial inoculation of the 25 different cell-types they found, including epidermal, endodermal, cortical and stem cells, and so forth. Interestingly, they identified an upregulation of genes involved in cell wall modifications and vesicle transport in root hairs, likely linked to the formation of curling root hairs associated with rhizobial infections. On the other hand, Ye et al. [70] focused their research only on mature nodule cells, identifying 13 spatially and functionally interconnected cell clusters that included apical, vascular and parenchyma cells, but also discovered functional clusters with diverse roles in the symbiotic process, including pre-infected, infected or nitrogen fixation clusters, among others. The authors revealed a diverse distribution of synthesis, transportation and metabolism of nutrients and substances across the different nodule cell-types; describing for example how symbiotic cells primarily perform nitrogen fixation, while un-symbiotic cells maintain homeostasis and provide energy for symbiotic cells, while surrounding them. Curiously, two unknown clusters were reported, because of their lack of specific marker genes. Later, other publications about single-cell resolution research on nodule development were released, employing diverse model legumes [60, 72–74]. They generated new cell atlases classifying major cell-types in both root and nodules and revealed a complex spatial compartmentalization of biochemical reactions during nitrogen fixation; particularly, Liu and colleagues analyzed two different nodule stages, and included GUS staining and RNA in situ hybridization assays to validate their observations, identifying diverse expression patterns and cell-type frequencies according to the nodule stage [75].

Serrano and collaborators [76] presented the first single-cell resolution study of the AM symbiosis, using *M. truncatula* and *R. irregularis* interaction as model. Through the integration of ST and snRNA-seq, they generated a map showing simultaneous plant and fungal transcriptomes during the symbiosis, in which they unravel a coordinated gene regulation between them. They analyzed the expression of AM-responsive genes reported in literature, inherent to each stage of colonization, through the different obtained clusters. They report a symbiosis-specific upregulation of several genes in both plant and fungus, identifying previously known and unknown transcripts and cell-type specific responses to the symbiosis. Among those, they observed the localized expression of five phosphate, three ammonium and two sugar transporters in a symbiosis-responsive cell cluster; which correlates with the continuous exchange of nutrients that occurs between the partners. The authors report as a major limitation of the study the low resolution of ST techniques, which do not reach the cell size, leading to a less accurate clustering [61].

On the other hand, three cell-resolution atlases of Arabidopsis leaves infected by *Pseudomonas syringae* have been released until now [77–79], revealing the complexity and heterogeneity of the response to bacterial infection among the different cell-types, showing coexistent cell populations that exhibit opposite states: immune and susceptible. Nobori et al. [79] introduced snMultiome in plant-pathogen research, a novel approach combining snRNA-seq and snATAC-seq (single-nucleus Assay for Transposase Accessible Chromatin sequencing), which allowed them to study single-cell transcriptome and epigenome in leaf cells responding to bacteria. To complement their results, they performed MERFISH, which allowed them to model the spatial heterogeneity of the plant immune response associated with pathogen distribution. Furthermore, Saarenpää et al. [80] introduced another innovative spatial transcriptomics technology to study *P. syringae* infection: Spatial metatranscriptomics (SmT), a sequencing-based approach that simultaneously considers the heterogeneity of not only the plant cells but the bacterial and fungal communities that interact with them, characterizing the microbiome signals from the tissues with a microscopic resolution.

Several publications studying the same plant-microbe interaction have yielded different or even opposite results regarding clustering of plant cell-types and how they respond to the symbiotic interaction. These differences could be attributed to the scarcity of published literature and the complexity of the data obtained. Additionally, the diversity on cell/nuclei isolation protocols, library preparation techniques and sequencing platforms available, among other factors, may have introduced biases into their findings. In summary, there is a low consensus and

correlation among experiments conducted by the different laboratories, as a result of the early stages of employing single-cell methodologies in the plant field. Indeed, the majority of the results obtained in the publications that are described here (Table 1) comprise high-resolution gene atlases, which give a spatial sense of the different cell-type clusters within a plant-microbe interaction but, above all, are valuable and powerful resources that will empower researchers to answer, or at least ask, new biological questions. As a common goal to coordinate efforts in generating plant single-cell atlases, The Plant Cell Atlas aims to serve as a community asset providing an exhaustive portrayal of diverse plant cell types. It will merge detailed location data on nucleic acids, proteins, and metabolites within plant cells at a high resolution, with guidelines for common practices and for sharing results and raw data [55, 81] and comprehensive datasets for multiple plant species [82].

Leveraging scRNA-seq to shed new light on AM development and regulation

In summary, plant single-cell NGS offers a comprehensive, but complex, analysis of thousands of individual cells, organizing them based on their statistical proximity. However, there still remains a diffused degree of scepticism regarding the validity of newly formed insights or hypotheses from this data that could go beyond the mere application of a "new" technique and the creation of an extra gene expression atlas [61]. Nevertheless, understanding the molecular bases of a highly complex tissue demands a high-resolution technique: this is particularly important when two distinct organisms interact and modulate their transcriptome depending on specific time and space. In the case of AM symbiosis, several long-lasting and new biological questions could arise and, eventually, be answered through these new approaches; here we discuss some of them: from the regulation of arbuscular development and degeneration, to the control of nutrient sensing in arbusculated cells and the identification of key allelic variants shaping plant responsiveness to AM fungi.

One of the biggest open questions concerns the molecular underpinnings and genetic regulation governing arbuscule turnover and degeneration. This phenomenon seems to be tightly regulated by plants, and suppressive genetic screenings pinpointed a MYB-like transcription factor that orchestrates this process [83, 84]. Nevertheless, the transcriptomic blueprint governing the short lifespan of arbuscules remains elusive, and the potential existence of feedback-loop control mechanisms remains unexplored. The ability to sequence the complete transcriptomes of plant cells harbouring arbuscules at various developmental stages, and link it to the specific fungal counterpart, or the access to mutant lines that are affected in different steps of arbuscule formation [85–87], will be indispensable for elucidating the molecular checkpoints that govern this intricate process.

The same approach could help us disentangling the molecular mechanisms governing the mutual recognition between AM fungi and plants during the development of arbuscules. Current knowledge has partially revealed the complexity of plant-fungus communication in the presymbiotic phase, where fungal short-chain chitin oligomers (COs) trigger plant nuclear and cytosolic calcium spiking and initiate a specific symbiosis [9, 88], but much less is known regarding the arbuscular phase. Recent studies have identified several kinases implicated in this process across various plant species that, together with fungal effectors [89] and miRNAs [90, 91] that are delivered into plant cells, could play a role during this step of the interaction [92–96]. Moreover, cell wall-associated kinases (WAKs), triggered by pectin disassembling, seem to play a pivotal role in tuning plant defence responses upon fungal growth [92, 93]. It appears clear that AM fungal intraradical growth represents a process at the boundary between plant defence suppression and precise activation of defence-related pathways, eventually leading and mediating the arbuscule degeneration. Currently, we know at least three different levels of modulation of plant defence mechanisms during the colonization by AM fungi: at local root level both a suppression of cellular immunity is necessary for hyphal growth, counterbalanced by specific activation of plant defence genes, such as chitinases and pathogenesis-related genes. At the systemic level, AM symbiosis often leads to priming effects [94, 95] and increasing our resolution on cells will indeed be instrumental in deciphering this conundrum.

Another open question that has been partially addressed regards the mechanisms by which plants can perceive cellular phosphate concentration and, consequently, induce or repress arbuscular mycorrhizal. Indeed PHOSPHATE STARVATION RESPONSE 1 (PHR1) is a master transcription factor regulating plant phosphate starvation responses and is necessary for the transcription of several AM-inducible genes [96]. This has been demonstrated in different plant species, but the key metabolites perceived by plants allowing to sense Pi concentration and modulate AM development are still not known. Hints come from *Arabidopsis thaliana* and yeast, where inositol phosphate (IP7/IP8) mirrors phosphate levels and mediates PHR1 sequestration by proteins containing the SPX domain [97–99]. It is foreseeable that plant single-cell metabolomics [100] and proteomics [101, 102] could reveal key metabolites and proteins differentially accumulating in plant cells hosting arbuscules at different stages and mediating plant cell nutrient perception both at local and systemic levels. This would increase our knowledge of the links between plant-fungal recognition and plant nutrient sensing where, so far,

only a few actors, such as NODULATION SIGNALING PATHWAY 1 and 2 (NSP1/2), have been described [5].

Finally, single-cell atlases generated from mycorrhizal roots will be instrumental in selecting candidate genes from Genome Wide Association Studies, similarly to what has been done for maize genomics, where a detailed transcriptome atlas of the developing maize ear inflorescence allowed the identification of potential loci linked to ear yield traits [103]. Several recent studies have attempted to unveil the genetic base of variation in plant growth responsiveness to AM fungi. This is a challenging goal, being plant responsiveness to AM fungi highly influenced by the environment. Therefore, only through the integration of multiple approaches and datasets, we will be able to discover genes and alleles underlying colonization levels or growth effects upon mycorrhization, beyond the ones that have already been identified [104–106].

Moving to more specific aspects of colonized cells, transmission electron microscopy has revealed interesting changes in both nuclear structure (chromatin moves to a very loose organization) and plastid morphology (stromules abundance) depending on the colonization phase [15, 107, 108]. Single-cell ATAC combined with RNA-seq could help to detect potential epigenetic events controlling nuclear features, as already identified in nematode-infected cells [109]. Another still open question concerns the signal(s) from the root meristem which block the AM growth. It is known that shoot apical meristem activates a sort of resistance to viruses [110], suggesting the potential presence of a common mechanism which makes stem cells resistant to exogenous microorganisms. On the other hand, the presence of AM fungi stimulates the production of new root meristems, increasing the branching, but again the specific activation site is unknown.

Altogether, due to the available genetics and molecular tools, most of our current knowledge on the AM symbiosis is plant-centric. In addition to the many questions that we have identified, single-cell level sequencing should surely help to better discover the mechanisms which underlie the morphogenesis of the symbiotic fungus, starting from the hyphopodium and the penetrating intracellular not-branched hyphae till the arbuscules or the vesicles. In the meantime, it could provide information on the saprotrophic potentialities of the fungus and on its use of mineral nutrients for its own metabolism. New knowledge could therefore lead to a more balanced information on the partners, allowing researchers to look at the interaction also from the fungal part. This could eventually lead to changing our ideas on the biology of the association, challenging the concept of mutualism for a more complex opportunistic relationship.

Conclusions

Decades of research on AM symbiosis and the efforts of an always growing scientific community have allowed us to disentangle many aspects of the intricate plant-fungal interactions. AM symbiosis is unique for the intimate contact of their partners and for its features which are surprisingly constant notwithstanding the huge biodiversity of the organisms involved [111]. This stability probably mirrors the antiquity of this alliance, suggesting that plant speciation occurred after AM establishment. The process of AM fungi accompanying most plants during evolution might have caused a strong link between organ development, especially of roots, and AM fungal presence.

It is evident that our understanding across multiple fields in biology, including plant research, has deeply advanced since we have gained access to single-cell resolution. Particularly, the ever expanding sequencing technologies could shed some light on the profound cellular heterogeneity inherent in this intimate plant-fungal interaction, offering new insights into the dynamic complexities of symbiotic relationships across evolution and within different ecosystems. The recent publication of the first AM symbiosis study using snRNAseq and ST, in which the authors generated an atlas showing plant and fungal transcriptomes simultaneously throughout their symbiotic relationship (72), is just the starting point in disentangling the molecular bases of this complex process with single-cell resolution. Certainly, these new technologies combined with the emerging ones in the coming years may address the questions that still lack answers and, in turn, promote the generation of new hypotheses.

Abbreviations

AM	Arbuscular Mycorrhizae/Mycorrhizal
MYA	Million Years Ago
scRNA-seq	Single-cell RNA sequencing
SL	Strigolactones
COs	Short-chain Chitin Oligomers
PCR	Polymerase Chain Reaction
GUS	β-glucuronidase
RT-qPCR	Real Time quantitative PCR
Pi	Inorganic Phosphate
RNA-seq	RNA sequencing
mRNA	Messenger RNA
snRNA-seq	Single-nucleus RNA sequencing
NGS	Next-generation sequencing
ST	Spatial Transcriptomics, LCM: Laser Capture Microdissection
MERFISH	Multiplexed Error Robust Fluorescence In Situ Hybridization
ATAC-seq	Assay for Transposase Accessible Chromatin sequencing
SmT	Spatial metatranscriptomics
WAKs	Cell Wall-Associated Kinases

Acknowledgements

We apologize in advance to all investigators whose research could not be appropriately cited owing to space limitations.

Author contributions

M.G., P.B and S.C.S. wrote the main manuscript text and S.C.S. prepared figures and tables. All authors reviewed the manuscript.

Funding
Research in MG's laboratory has been supported by the European Union - NextGenerationEU (PRIN PNRR prot. P2022WL8TS and 2021 STARS Grants@ Unipd programme P-NICHE) the University of Padova (Progetti di Ricerca Dipartimentali - PRID grant no. BIRD214519) and the University of Torino (Ricerca Locale 2023).

Data availability
No datasets were generated or analysed during the current study.

Declarations

Competing interests
The authors declare no competing interests.

Received: 2 May 2024 / Accepted: 11 July 2024
Published online: 17 August 2024

References

1. Bonfante P. The future has roots in the past: the ideas and scientists that shaped mycorrhizal research. New Phytol. 2018;220:982–95.
2. Tedersoo L, Bahram M, Zobel M. How mycorrhizal associations drive plant population and community biology. Science. 2020;367:eaba1223.
3. Zhang X, Wang L, Ma F, Yang J, Su M. Effects of arbuscular mycorrhizal fungi inoculation on carbon and nitrogen distribution and grain yield and nutritional quality in rice (*Oryza sativa* L). J Sci Food Agric. 2017;97:2919–25.
4. Chialva M, Patono DL, de Souza LP, Novero M, Vercellino S, Maghrebi M, et al. The mycorrhizal root-shoot axis elicits *Coffea arabica* growth under low phosphate conditions. New Phytol. 2023;239:271–85.
5. Li X-R, Sun J, Albinsky D, Zarrabian D, Hull R, Lee T, et al. Nutrient regulation of lipochitooligosaccharide recognition in plants via NSP1 and NSP2. Nat Commun. 2022;13:6421.
6. Giovannetti M, Binci F, Navazio L, Genre A. Nonbinary fungal signals and calcium-mediated transduction in plant immunity and symbiosis. New Phytol. 2024;241:1393–400.
7. Lanfranco L, Bonfante P. Lessons from arbuscular mycorrhizal fungal genomes. Curr Opin Microbiol. 2023;75:102357.
8. Besserer A, Puech-Pagès V, Kiefer P, Gomez-Roldan V, Jauneau A, Roy S, et al. Strigolactones stimulate arbuscular mycorrhizal fungi by activating mitochondria. PLoS Biol. 2006;4:e226.
9. Genre A, Chabaud M, Balzergue C, Puech-Pagès V, Novero M, Rey T, et al. Short-chain chitin oligomers from arbuscular mycorrhizal fungi trigger nuclear Ca^{2+} spiking in *Medicago truncatula* roots and their production is enhanced by strigolactone. New Phytol. 2013;198:190–202.
10. Bonfante P, Genre A. Mechanisms underlying beneficial plant–fungus interactions in mycorrhizal symbiosis. Nat Commun. 2010;1:48.
11. Bonfante P. At the interface between mycorrhizal Fungi and plants: the Structural Organization of Cell Wall, plasma membrane and Cytoskeleton. 2001. p. 45–61.
12. Bonfante-Fasolo P, Faccio A, Perotto S, Schubert A. Correlation between chitin distribution and cell wall morphology in the mycorrhizal fungus Glomus Versiforme. Mycol Res. 1990;94:157–65.
13. Pumplin N, Harrison MJ. Live-cell imaging reveals periarbuscular membrane domains and organelle location in Medicago truncatula roots during arbuscular mycorrhizal symbiosis. Plant Physiol. 2009;151:809–19.
14. Balestrini R, Hahn MG, Faccio A, Mendgen K, Bonfante P. Differential localization of Carbohydrate epitopes in Plant cell walls in the Presence and absence of Arbuscular Mycorrhizal Fungi. Plant Physiol. 1996;111:203–13.
15. Balestrini R, Berta G, Bonfante P. The plant nucleus in mycorrhizal roots: positional and structural modifications. Biol Cell. 1992;75:235–43.
16. Bianciotto V, Genre A, Jargeat P, Lumini E, Bécard G, Bonfante P. Vertical transmission of endobacteria in the arbuscular mycorrhizal fungus Gigaspora margarita through generation of vegetative spores. Appl Environ Microbiol. 2004;70:3600–8.
17. Uehling J, Salvioli A, Amses K, Partida Martinez L, Bonito G, Bonfante P. Bacterial Endosymbionts of Mucoromycota Fungi: Diversity and Function of their Interactions. 2023. pp. 177–205.
18. Smith SE, Smith FA. Structure and function of the interfaces in biotrophic symbioses as they relate to nutrient transport. New Phytol. 1990;114:1–38.
19. Jefferson RA, Kavanagh TA, Bevan MW. GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. 1987;6:3901–7.
20. Rausch C, Daram P, Brunner S, Jansa J, Laloi M, Leggewie G, et al. A phosphate transporter expressed in arbuscule-containing cells in potato. Nature. 2001;414:462–70.
21. Harrison MJ, Dewbre GR, Liu J. A phosphate transporter from Medicago truncatula involved in the acquisition of phosphate released by arbuscular mycorrhizal fungi. Plant Cell. 2002;14:2413–29.
22. Paszkowski U, Kroken S, Roux C, Briggs SP. Rice phosphate transporters include an evolutionarily divergent gene specifically activated in arbuscular mycorrhizal symbiosis. Proc Natl Acad Sci. 2002;99:13324–9.
23. Volpe V, Giovannetti M, Sun X-G, Fiorilli V, Bonfante P. The phosphate transporters LjPT4 and MtPT4 mediate early root responses to phosphate status in non mycorrhizal roots. Plant Cell Environ. 2016;39:660–71.
24. Genre A, Chabaud M, Faccio A, Barker DG, Bonfante P. Prepenetration apparatus assembly precedes and predicts the colonization patterns of arbuscular mycorrhizal fungi within the root cortex of both Medicago truncatula and Daucus carota. Plant Cell. 2008;20:1407–20.
25. Genre A, Chabaud M, Timmers T, Bonfante P, Barker DG. Arbuscular mycorrhizal fungi elicit a novel intracellular apparatus in Medicago truncatula root epidermal cells before infection. Plant Cell. 2005;17:3489–99.
26. Genre A, Ivanov S, Fendrych M, Faccio A, Zársky V, Bisseling T, et al. Multiple exocytotic markers accumulate at the sites of perifungal membrane biogenesis in arbuscular mycorrhizas. Plant Cell Physiol. 2012;53:244–55.
27. Ivanov S, Harrison MJ. Receptor-associated kinases control the lipid provisioning program in plant-fungal symbiosis. Science. 2024;383:443–8.
28. Balestrini R, Gómez-Ariza J, Lanfranco L, Bonfante P. Laser microdissection reveals that transcripts for five plant and one fungal phosphate transporter genes are contemporaneously present in arbusculated cells. Mol Plant-Microbe Interact MPMI. 2007;20:1055–62.
29. Gomez SK, Harrison MJ. Laser microdissection and its application to analyze gene expression in arbuscular mycorrhizal symbiosis. Pest Manag Sci. 2009;65:504–11.
30. Gomez SK, Javot H, Deewatthanawong P, Torres-Jerez I, Tang Y, Blancaflor EB, et al. Medicago truncatula and Glomus intraradices gene expression in cortical cells harboring arbuscules in the arbuscular mycorrhizal symbiosis. BMC Plant Biol. 2009;9:10.
31. Gómez-Ariza J, Balestrini R, Novero M, Bonfante P. Cell-specific gene expression of phosphate transporters in mycorrhizal tomato roots. Biol Fertil Soils. 2009;45:845–53.
32. Guether M, Balestrini R, Hannah M, He J, Udvardi MK, Bonfante P. Genome-wide reprogramming of regulatory networks, transport, cell wall and membrane biogenesis during arbuscular mycorrhizal symbiosis in Lotus japonicus. New Phytol. 2009;182:200–12.
33. Hogekamp C, Küster H. A roadmap of cell-type specific gene expression during sequential stages of the arbuscular mycorrhiza symbiosis. BMC Genomics. 2013;14:306.
34. Gaude N, Schulze WX, Franken P, Krajinski F. Cell type-specific protein and transcription profiles implicate periarbuscular membrane synthesis as an important carbon sink in the mycorrhizal symbiosis. Plant Signal Behav. 2012;7:461–4.
35. Gaude N, Bortfeld S, Duensing N, Lohse M, Krajinski F. Arbuscule-containing and non-colonized cortical cells of mycorrhizal roots undergo extensive and specific reprogramming during arbuscular mycorrhizal development. Plant J Cell Mol Biol. 2012;69:510–28.
36. Giovannetti M, Balestrini R, Volpe V, Guether M, Straub D, Costa A, et al. Two putative-aquaporin genes are differentially expressed during arbuscular mycorrhizal symbiosis in *Lotus japonicus*. BMC Plant Biol. 2012;12:186.
37. Hogekamp C, Arndt D, Pereira PA, Becker JD, Hohnjec N, Küster H. Laser microdissection unravels cell-type-specific transcription in arbuscular mycorrhizal roots, including CAAT-box transcription factor gene expression correlating with fungal contact and spread. Plant Physiol. 2011;157:2023–43.
38. Guether M, Neuhäuser B, Balestrini R, Dynowski M, Ludewig U, Bonfante P. A mycorrhizal-specific ammonium transporter from Lotus japonicus acquires nitrogen released by arbuscular mycorrhizal fungi. Plant Physiol. 2009;150:73–83.
39. Giovannetti M, Tolosano M, Volpe V, Kopriva S, Bonfante P. Identification and functional characterization of a sulfate transporter induced by both sulfur starvation and mycorrhiza formation in Lotus japonicus. New Phytol. 2014;204:609–19.

40. Meier-Ruge W, Bielser W, Remy E, Hillenkamp F, Nitsche R, Unsöld R. The laser in the Lowry technique for microdissection of freeze-dried tissue slices. Histochem J. 1976;8:387–401.
41. Asano T, Masumura T, Kusano H, Kikuchi S, Kurita A, Shimada H, et al. Construction of a specialized cDNA library from plant cells isolated by laser capture microdissection: toward comprehensive analysis of the genes expressed in the rice phloem. Plant J Cell Mol Biol. 2002;32:401–8.
42. Tisserant E, Kohler A, Dozolme-Seddas P, Balestrini R, Benabdellah K, Colard A, et al. The transcriptome of the arbuscular mycorrhizal fungus Glomus intraradices (DAOM 197198) reveals functional tradeoffs in an obligate symbiont. New Phytol. 2012;193:755–69.
43. Domingo G, Vannini C, Bracale M, Bonfante P. Proteomics as a tool to decipher plant responses in arbuscular mycorrhizal interactions: a meta-analysis. Proteomics. 2023;23:e2200108.
44. Nagata T, Takebe I. Cell wall regeneration and cell division in isolated tobacco mesophyll protoplasts. Planta. 1970;92:301–8.
45. Rich-Griffin C, Stechemesser A, Finch J, Lucas E, Ott S, Schäfer P. Single-cell transcriptomics: a high-Resolution Avenue for Plant Functional Genomics. Trends Plant Sci. 2020;25:186–97.
46. Jean-Baptiste K, McFaline-Figueroa JL, Alexandre CM, Dorrity MW, Saunders L, Bubb KL, et al. Dynamics of Gene expression in single Root cells of Arabidopsis thaliana. Plant Cell. 2019;31:993–1011.
47. Shulse CN, Cole BJ, Ciobanu D, Lin J, Yoshinaga Y, Gouran M, et al. High-throughput single-cell transcriptome profiling of plant cell types. Cell Rep. 2019;27:2241–e22474.
48. Bawa G, Liu Z, Yu X, Qin A, Sun X. Single-cell RNA sequencing for Plant Research: insights and possible benefits. Int J Mol Sci. 2022;23:4497.
49. Shaw R, Tian X, Xu J. Single-cell transcriptome analysis in plants: advances and challenges. Mol Plant. 2021;14:115–26.
50. Conde D, Triozzi PM, Balmant KM, Doty AL, Miranda M, Boullosa A, et al. A robust method of nuclei isolation for single-cell RNA sequencing of solid tissues from the plant genus Populus. PLoS ONE. 2021;16:e0251149.
51. Charlot F, Goudounet G, Nogué F, Perroud P-F. Physcomitrium patens protoplasting and protoplast transfection. Methods Mol Biol Clifton NJ. 2022;2464:3–19.
52. Birnbaum K, Jung JW, Wang JY, Lambert GM, Hirst JA, Galbraith DW, et al. Cell type-specific expression profiling in plants via cell sorting of protoplasts from fluorescent reporter lines. Nat Methods. 2005;2:615–9.
53. Farmer A, Thibivilliers S, Ryu KH, Schiefelbein J, Libault M. Single-nucleus RNA and ATAC sequencing reveals the impact of chromatin accessibility on gene expression in Arabidopsis roots at the single-cell level. Mol Plant. 2021;14:372–83.
54. Guillotin B, Rahni R, Passalacqua M, Mohammed MA, Xu X, Raju SK, et al. A pan-grass transcriptome reveals patterns of cellular divergence in crops. Nature. 2023;617:785–91.
55. Grones C, Eekhout T, Shi D, Neumann M, Berg LS, Ke Y, et al. Best practices for the execution, analysis, and data storage of plant single-cell/nucleus transcriptomics. Plant Cell. 2024;36:812–28.
56. Svensson V, Vento-Tormo R, Teichmann SA. Exponential scaling of single-cell RNA-seq in the past decade. Nat Protoc. 2018;13:599–604.
57. Jiang L, Yang H, Cheng W, Ni Z, Xiang N. Droplet microfluidics for CTC-based liquid biopsy: a review. Analyst. 2023;148:203–21.
58. Zheng GXY, Terry JM, Belgrader P, Ryvkin P, Bent ZW, Wilson R, et al. Massively parallel digital transcriptional profiling of single cells. Nat Commun. 2017;8:14049.
59. Rosenberg AB, Roco CM, Muscat RA, Kuchina A, Sample P, Yao Z, et al. Single-cell profiling of the developing mouse brain and spinal cord with split-pool barcoding. Science. 2018;360:176–82.
60. Pereira WJ, Boyd J, Conde D, Triozzi PM, Balmant KM, Dervinis C, et al. The single-cell transcriptome program of nodule development cellular lineages in Medicago truncatula. Cell Rep. 2024;43:113747.
61. Adema K, Schon MA, Nodine MD, Kohlen W. Lost in space: what single-cell RNA sequencing cannot tell you. Trends Plant Sci. 2024;S1360-1385(24)00066 – 9.
62. Giacomello S. A new era for plant science: spatial single-cell transcriptomics. Curr Opin Plant Biol. 2021;60:102041.
63. Chen KH, Boettiger AN, Moffitt JR, Wang S, Zhuang X. Spatially resolved, highly multiplexed RNA profiling in single cells. Science. 2015;348:aaa6090.
64. Yin R, Xia K, Xu X. Spatial transcriptomics drives a new era in plant research. Plant J Cell Mol Biol. 2023;116:1571–81.
65. You Y, Fu Y, Li L, Zhang Z, Jia S, Lu S, Ren W, Liu Y, Xu Y, Liu X, Jiang F, Peng G, Sampath Kumar A, Ritchie ME, Liu X, Tian L. Systematic comparison of sequencing-based spatial transcriptomic methods. Nat Methods. 2024 July 4. https://doi.org/10.1038/s41592-024-02325-3. PMID: 38965443
66. Liu C, Leng J, Li Y, Ge T, Li J, Chen Y, et al. A spatiotemporal atlas of organogenesis in the development of orchid flowers. Nucleic Acids Res. 2022;50:9724–37.
67. Moreno-Villena JJ, Zhou H, Gilman IS, Tausta SL, Cheung CYM, Edwards EJ. Spatial resolution of an integrated C4 + CAM photosynthetic metabolism. Sci Adv. 2022;8:eabn2349.
68. Tenorio Berrío R, Dubois M. Single-cell transcriptomics reveal heterogeneity in plant responses to the environment: a focus on biotic and abiotic interactions. J Exp Bot. 2024;erae107.
69. Bai Y, Liu H, Lyu H, Su L, Xiong J, Cheng Z-MM. Development of a single-cell atlas for woodland strawberry (Fragaria vesca) leaves during early Botrytis cinerea infection using single cell RNA-seq. Hortic Res. 2022;9:uhab055.
70. Ye Q, Zhu F, Sun F, Wang T-C, Wu J, Liu P, et al. Differentiation trajectories and biofunctions of symbiotic and un-symbiotic fate cells in root nodules of Medicago truncatula. Mol Plant. 2022;15:1852–67.
71. Cervantes-Pérez SA, Thibivilliers S, Laffont C, Farmer AD, Frugier F, Libault M. Cell-specific pathways recruited for symbiotic nodulation in the Medicago truncatula legume. Mol Plant. 2022;15:1868–88.
72. Sun B, Wang Y, Yang Q, Gao H, Niu H, Li Y, et al. A high-resolution transcriptomic atlas depicting nitrogen fixation and nodule development in soybean. J Integr Plant Biol. 2023;65:1536–52.
73. Cervantes-Pérez SA, Zogli P, Thibivilliers S, Tennant S, Hossain MS, Xu H et al. Single-cell resolution transcriptome atlases of soybean root organs reveal new regulatory programs controlling the nodulation process. Preprint at https://www.researchsquare.com/article/rs-2833917/v1 (2023).
74. Frank M, Fechete LI, Tedeschi F, Nadzieja M, Nørgaard MMM, Montiel J, et al. Single-cell analysis identifies genes facilitating rhizobium infection in Lotus japonicus. Nat Commun. 2023;14:7171.
75. Liu Z, Yang J, Long Y, Zhang C, Wang D, Zhang X, et al. Single-nucleus transcriptomes reveal spatiotemporal symbiotic perception and early response in Medicago. Nat Plants. 2023;9:1734–48.
76. Serrano K, Bezrutczyk M, Goudeau D, Dao T, O'Malley R, Malmstrom RR, et al. Spatial co-transcriptomics reveals discrete stages of the arbuscular mycorrhizal symbiosis. Nat Plants. 2024;10:673–88.
77. Delannoy E, Batardiere B, Pateyron S, Soubigou-Taconnat L, Chiquet J, Colcombet J, et al. Cell specialization and coordination in Arabidopsis leaves upon pathogenic attack revealed by scRNA-seq. Plant Commun. 2023;4:100676.
78. Zhu J, Lolle S, Tang A, Guel B, Kvitko B, Cole B, et al. Single-cell profiling of Arabidopsis leaves to Pseudomonas syringae infection. Cell Rep. 2023;42:112676.
79. Nobori T, Monell A, Lee TA, Zhou J, Nery J, Ecker JR. Time-resolved single-cell and spatial gene regulatory atlas of plants under pathogen attack. Preprint at https://www.biorxiv.org/content/https://doi.org/10.1101/2023.04.10.536170v2 (2023).
80. Saarenpää S, Shalev O, Ashkenazy H, Carlos V, Lundberg DS, Weigel D et al. Spatial metatranscriptomics resolves host-bacteria-fungi interactomes. Nat Biotechnol. 2023.
81. Fahlgren N, Kapoor M, Yordanova G, Papatheodorou I, Waese J, Cole B, et al. Toward a data infrastructure for the Plant Cell Atlas. Plant Physiol. 2023;191:35–46.
82. He Z, Luo Y, Zhou X, Zhu T, Lan Y, Chen D. scPlantDB: a comprehensive database for exploring cell types and markers of plant cell atlases. Nucleic Acids Res. 2024;52:D1629–38.
83. Volpe V, Dell'Aglio E, Giovannetti M, Ruberti C, Costa A, Genre A, et al. An AM-induced, MYB-family gene of *Lotus japonicus* (LjMAMI) affects root growth in an AM-independent manner. Plant J Cell Mol Biol. 2013;73:442–55.
84. Floss DS, Gomez SK, Park H-J, MacLean AM, Müller LM, Bhattarai KK, et al. A Transcriptional Program for Arbuscule Degeneration during AM Symbiosis is regulated by MYB1. Curr Biol. 2017;27:1206–12.
85. Floss DS, Levy JG, Lévesque-Tremblay V, Pumplin N, Harrison MJ. DELLA proteins regulate arbuscule formation in arbuscular mycorrhizal symbiosis. Proc Natl Acad Sci U S A. 2013;110:E5025–5034.
86. Kistner C, Winzer T, Pitzschke A, Mulder L, Sato S, Kaneko T, et al. Seven Lotus japonicus genes required for transcriptional reprogramming of the root during fungal and bacterial symbiosis. Plant Cell. 2005;17:2217–29.
87. Yano K, Yoshida S, Müller J, Singh S, Banba M, Vickers K, et al. CYCLOPS, a mediator of symbiotic intracellular accommodation. Proc Natl Acad Sci U S A. 2008;105:20540–5.
88. Binci F, Offer E, Crosino A, Sciascia I, Kleine-Vehn J, Genre A, et al. Spatially and temporally distinct Ca^{2+} changes in *Lotus japonicus* roots orient

fungal-triggered signalling pathways towards symbiosis or immunity. J Exp Bot. 2024;75:605–19.
89. Betz R, Heidt S, Figueira-Galán D, Langner T, Requena N. Alternative splicing regulation in plants by effectors of symbiotic arbuscular mycorrhizal fungi. Preprint at https://www.biorxiv.org/content/https://doi.org/10.1101/2023.09.20.558436v1 (2023).
90. Lanfranco L, Silvestri A, Ledford W, Fiorilli V, Votta C, Scerna A et al. Cross-kingdom RNAi adds a new layer of communication to the arbuscular mycorrhizal symbiosis. Preprint at https://www.researchsquare.com/article/rs-3423345/v1 (2023).
91. Ledford WC, Silvestri A, Fiorilli V, Roth R, Rubio-Somoza I, Lanfranco L. A journey into the world of small RNAs in the arbuscular mycorrhizal symbiosis. New Phytol. 2024;242:1534–44.
92. Giovannetti M, Genre A. Walking on a tightrope: cell wall-associated kinases act as sensors and regulators of immunity and symbiosis. New Phytol. 2024. https://doi.org/10.1111/nph.19634.
93. Zhang X, Jia S, He Y, Wen J, Li D, Yang W, et al. Wall-associated kinase GhWAK13 mediates arbuscular mycorrhizal symbiosis and Verticillium wilt resistance in cotton. New Phytol. 2023. https://doi.org/10.1111/nph.19468.
94. Fiorilli V, Vannini C, Ortolani F, Garcia-Seco D, Chiapello M, Novero M, et al. Omics approaches revealed how arbuscular mycorrhizal symbiosis enhances yield and resistance to leaf pathogen in wheat. Sci Rep. 2018;8:9625.
95. Rivero J, Lidoy J, Llopis-Giménez Á, Herrero S, Flors V, Pozo MJ. Mycorrhizal symbiosis primes the accumulation of antiherbivore compounds and enhances herbivore mortality in tomato. J Exp Bot. 2021;72:5038–50.
96. Rouached H, Secco D, Arpat B, Poirier Y. The transcription factor PHR1 plays a key role in the regulation of sulfate shoot-to-root flux upon phosphate starvation in Arabidopsis. BMC Plant Biol. 2011;11:19.
97. Puga MI, Mateos I, Charukesi R, Wang Z, Franco-Zorrilla JM, de Lorenzo L, et al. SPX1 is a phosphate-dependent inhibitor of phosphate starvation response 1 in Arabidopsis. Proc Natl Acad Sci U S A. 2014;111:14947–52.
98. Osorio MB, Ng S, Berkowitz O, De Clercq I, Mao C, Shou H, et al. SPX4 acts on PHR1-Dependent and -independent regulation of shoot Phosphorus Status in Arabidopsis. Plant Physiol. 2019;181:332–52.
99. Secco D, Wang C, Shou H, Whelan J. Phosphate homeostasis in the yeast Saccharomyces cerevisiae, the key role of the SPX domain-containing proteins. FEBS Lett. 2012;586:289–95.
100. Pandian K, Matsui M, Hankemeier T, Ali A, Okubo-Kurihara E. Advances in single-cell metabolomics to unravel cellular heterogeneity in plant biology. Plant Physiol. 2023;193:949–65.
101. Clark NM, Elmore JM, Walley JW. To the proteome and beyond: advances in single-cell omics profiling for plant systems. Plant Physiol. 2022;188:726–37.
102. Gatto L, Aebersold R, Cox J, Demichev V, Derks J, Emmott E, et al. Initial recommendations for performing, benchmarking and reporting single-cell proteomics experiments. Nat Methods. 2023;20:375–86.
103. Xu X, Crow M, Rice BR, Li F, Harris B, Liu L, et al. Single-cell RNA sequencing of developing maize ears facilitates functional analysis and trait candidate gene discovery. Dev Cell. 2021;56:557–e5686.
104. Huang R, Li Z, Mao C, Zhang H, Sun Z, Li H, et al. Natural variation at OsCERK1 regulates arbuscular mycorrhizal symbiosis in rice. New Phytol. 2020;225:1762–76.
105. Han R, Yang Z, Wang C, Zhu S, Tang G, Shen X, et al. Wild species rice OsCERK1DY-mediated arbuscular mycorrhiza symbiosis boosts yield and nutrient use efficiency in rice breeding. Mol Breed New Strateg Plant Improv. 2024;44:22.
106. Ramírez-Flores MR, Perez-Limon S, Li M, Barrales-Gamez B, Albinsky D, Paszkowski U, et al. The genetic architecture of host response reveals the importance of arbuscular mycorrhizae to maize cultivation. eLife. 2020;9:e61701.
107. Lohse S, Schliemann W, Ammer C, Kopka J, Strack D, Fester T. Organization and metabolism of plastids and mitochondria in arbuscular mycorrhizal roots of Medicago truncatula. Plant Physiol. 2005;139:329–40.
108. Fester T, Strack D, Hause B. Reorganization of tobacco root plastids during arbuscule development. Planta. 2001;213:864–8.
109. Brown IK, Dyjack N, Miller MM, Krovi H, Rios C, Woolaver R, et al. Single cell analysis of host response to helminth infection reveals the clonal breadth, heterogeneity, and tissue-specific programming of the responding CD4 + T cell repertoire. PLoS Pathog. 2021;17:e1009602.
110. Hoffmann G, Incarbone M. A resilient bunch: stem cell antiviral immunity in plants. New Phytol. 2024;241:1415–20.
111. Brundrett MC, Tedersoo L. Evolutionary history of mycorrhizal symbioses and global host plant diversity. New Phytol. 2018;220:1108–15.
112. Tang B, Feng L, Hulin MT, Ding P, Ma W. Cell-type-specific responses to fungal infection in plants revealed by single-cell transcriptomics. Cell Host Microbe. 2023;31:1732–e17475.
113. Cao Y, Ma J, Han S, Hou M, Wei X, Zhang X, et al. Single-cell RNA sequencing profiles reveal cell type-specific transcriptional regulation networks conditioning fungal invasion in maize roots. Plant Biotechnol J. 2023;21:1839–59.
114. Liu Z, Kong X, Long Y, Liu S, Zhang H, Jia J, et al. Integrated single-nucleus and spatial transcriptomics captures transitional states in soybean nodule maturation. Nat Plants. 2023;9:515–24.
115. Liang X, Ma Z, Ke Y, Wang J, Wang L, Qin B, et al. Single-cell transcriptomic analyses reveal cellular and molecular patterns of rubber tree response to early powdery mildew infection. Plant Cell Environ. 2023;46:2222–37.

Publisher's Note